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Structure of the *CAC1* Gene and in Situ Characterization of Its Expression[1]

The *Arabidopsis thaliana* Gene Coding for the Biotin-Containing Subunit of the Plastidic Acetyl-Coenzyme A Carboxylase

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The *CAC1* gene of *Arabidopsis thaliana* that codes for the biotin carboxyl-carrier subunit of the heteromeric acetyl-coenzyme A carboxylase was isolated and sequenced. *CAC1* is a single-copy gene interrupted by six introns. Subcellular immunogold labeling indicates that the biotin carboxyl-carrier subunit is localized in the stroma of the plastids and chloroplasts. The *CAC1* mRNA accumulates throughout developing embryos and ovules of siliques at a time of rapid growth and oil accumulation (7 d after flowering), but is present at much lower levels in wall cells and central septal cells of the silique. Immunolocalization studies show that the pattern of accumulation of the biotin carboxyl-carrier subunit within the siliques and leaves is similar to that of the *CAC1* mRNA. These observations indicate that the cellular pattern of biotin carboxyl-carrier protein accumulation in the developing silique may be determined by the transcriptional activity of the *CAC1* gene.

The first committed reaction of de novo fatty acid biosynthesis, the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, is catalyzed by ACCase (acetyl-CoA: carbon-dioxide ligase [ADP-forming], EC 6.4.1.2). This biotin-containing enzyme is at a critical control point for fatty acid synthesis and has been shown to regulate the flux through this pathway in animals, yeast, bacteria, and chloroplasts (Vagelos, 1971; Lane et al., 1974; Wakil et al., 1983; Hasslacher et al., 1993; Li and Cronan, 1993; Ohlrogge et al., 1993).

In contrast to most other organisms, plants utilize malonyl-CoA not only for fatty acid biosynthesis but also for the synthesis of a variety of phytochemicals, including flavonoids, stilbenoids, malonated D-amino acids, and malonated ACC, and for the elongation of long-chain fatty acids (Conn, 1981; Nikolau et al., 1984). Most of these specialized phytochemicals are synthesized in the cytosol (Conn, 1981; Nikolau et al., 1984), whereas de novo fatty acid biosynthesis is plastidic (Ohlrogge et al., 1979; Harwood, 1988). This compartmentation of the synthesis of molecules derived from malonyl-CoA led to the hypothesis that at least two isozymes of ACCase, a cytosolic isozyme and a plastidic isozyme, are present in plants, enabling the independent generation of malonyl-CoA in each of these subcellular compartments (Pollard and Stumpf, 1980; Nikolau et al., 1984).

Thus far, three structurally diverse forms of ACCase have been identified (reviewed by Lane et al., 1974; Choi et al., 1995). At least two of these forms occur in plants (Sasaki et al., 1995). A nondissociable, homomeric ACCase with a single type of biotin-containing polypeptide, ranging in size from 220 to 260 kD, is present in the cytosol of eukaryotic organisms such as yeast (Walid et al., 1992), animals (Lopez-Casillas et al., 1988), diatoms (Roessler and Ohlrogge, 1993), and plants (Egin-Buhler et al., 1980; Gornicki et al., 1993; Roesler et al., 1994; Schulte et al., 1994; Shorrosh et al., 1994; Yanai et al., 1995). This multifunctional polypeptide contains the biotin carboxylase, BCC, and carboxyltransferase domains. A homomeric ACCase of similar structure is also present in the plastids of some plant species, including maize (Egli et al., 1993) and wheat (Gornicki et al., 1994).

A heteromeric ACCase with several structural similarities to *Escherichia coli* ACCase (Guchhait et al., 1974; Kondo et al., 1991; Li and Cronan, 1992a, 1992b) is present in many species of plants (Kannangara and Stumpf, 1972; Sasaki et al., 1993; Alban et al., 1994, 1995; Konishi and Sasaki, 1994; Choi et al., 1995; Shorrosh et al., 1995). The quaternary structure of this plant heteromeric ACCase is not yet fully characterized. It has three known components: (a) a BCC subunit, to which biotin is covalently bound and which is nucleus-encoded (Choi et al., 1995); (b) biotin carboxylase, which carboxylates the BCC-bound biotin prosthetic group and is also encoded by a nuclear gene (Shorrosh et al., 1995); and (c) carboxyltransferase, which transfers a car-

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Abbreviations ACCase, acetyl-CoA carboxylase; BCC, biotin carboxyl-carrier; DAF, days after flowering; PAP, peroxidase-antiperoxidase.

boxyl group from BCC-bound biotin to acetyl-CoA, generating malonyl-CoA. This latter component is composed of two types of polypeptides, one plastid-encoded (Sasaki et al., 1993) and the other nucleus-encoded (Shorrosh et al., 1996; N. Nielson, GenBank accession number U40979).

This study reports the isolation and characterization of the nuclear *CAC1* (chloroplastic acetyl-CoA carboxylase) gene of *Arabidopsis thaliana* that encodes the BCC subunit of the heteromeric ACCase. We have used immunocytochemical procedures to directly confirm that the BCC subunit encoded by the *CAC1* gene is localized in the stroma of plastids. In situ hybridization and immunocytochemistry were used to determine the pattern of accumulation of the *CAC1* mRNA and the BCC subunit in siliques and leaves.

MATERIALS AND METHODS

Arabidopsis thaliana (L.) Heynh. (Columbia) seeds were germinated in sterile soil and plants were grown at 25°C under constant illumination. After the initial two flowers had opened, subsequent flowers were tagged with color-coded threads on the day they opened. Siliques were harvested 7 DAF. BCC antiserum was obtained as previously described (Choi et al., 1995). A genomic library of Arabidopsis (Voytas et al., 1990) was obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Isolation and Characterization of Macromolecules

Standard procedures were used for the isolation, analysis, and manipulation of nucleic acids (Sambrook et al., 1989). The Arabidopsis genomic library was screened by hybridization with the 730 bp that constituted the 3′-most fragment of the *CAC1* cDNA (Choi et al., 1995). Approximately 200,000 recombinant phages were grown on Petri plates and replicated to nitrocellulose membranes. The replica filters were incubated at 65°C in 6× SSC (1× SSC is 0.15 M NaCl and 15 mM sodium citrate, pH 7.0) with ^{32}P-labeled probe for 12 h. After hybridization, filters were washed at 65°C in 2× SSC and 0.5% SDS, and subsequently with 0.2× SSC and 0.1% SDS.

Double-stranded DNA templates were sequenced using an ABI 373A DNA sequencer (Applied Biosystems) at the Iowa State University Nucleic Acids Facility (Ames, IA). Both strands of all DNA fragments were sequenced at least two times. Primers used to sequence were the M13 reverse primer and the primers that matched sequences within the *CAC1* gene: PF1 (5′-GTAGTAAAGGAAGTGACCG); PF2 (5′-CTTTCCGTCTCTCTGCTAAGCC); PF3 (5′-ACCATCCCCACCTACTCCAG); PF4 (5′-ACTCCTCTGTTTGTGGTTC); PR1 (5′-CAATCCTTGTGGACTATC); PR2 (5′-ATTGGGAAGTGCGAGGAG);PR3(5′-TTAGCAGAGAGACGGAAAG); PR4 (5′-TTGGGTAAGGAACTCAGA); PR5 (5′-GCATAAGATGGTTGATTTGG); PR6 (5′-GTAGGAAGTGACGATTTGGC); and PR7 (5′-CCAAAGCACAGAGAATGA).

In Situ Techniques

In situ hybridization to RNA using paraffin-embedded sections was carried out as described previously (John et al., 1992; Wang et al., 1995). ^{35}S-labeled RNA probes (sense and antisense) were synthesized from a subclone consisting of the 730 nucleotides at the 3′ end of the *CAC1* cDNA (Choi et al., 1995). Slides with hybridized tissue sections were coated with nuclear track emulsion (Kodak NTB2), exposed for 1 to 4 d, and developed. Photographs were taken with a microscope (Orthoplan, Leitz, Wetzlar, Germany) using bright-field optics and Ectachrome film (Kodak). In situ hybridization results were repeated four times using two sets of plant materials that had been independently processed, all of which gave similar results.

For immunohistochemical subcellular localization studies, 2- to 3-mm segments of siliques were fixed in 2% (v/v) paraformaldehyde and 1% (v/v) glutaraldehyde in a 0.1 M phosphate buffer, pH 7.2. Fixation was initially at room temperature for 30 min under a low vacuum to enhance infiltration, and then for an additional 90 min at 4°C. Fixed segments were dehydrated in an increasing ethanol series and embedded in London Resin White. Sixty-nanometer-thin sections were placed on Formvar-coated (Electron Microscopy Sciences, Fort Washington, PA) nickel grids and allowed to dry for at least 12 h. Sections on grids were incubated at 37°C for 5 h in a moist chamber with either BCC antiserum (Choi et al., 1995) diluted 1:500 with TBS buffer (0.05 M Tris-HCl, pH 8.3–8.5, 0.85% [w/v] NaCl, 0.5% [w/v] BSA, 0.5% [v/v] normal goat serum) or preimmune serum diluted 1:500 with TBS buffer. After incubation, grids were washed with a stream of TBS buffer and then washed three times for 5 min each time in 25 μL of TBS buffer. Grids were carefully blotted dry and incubated for 5 h at room temperature in goat anti-rabbit IgG conjugated to 10-nm immunogold diluted 1:100 with TBS plus 0.1% (w/v) fish gelatin. Grids were then washed with a stream of distilled water and twice immersed for 10 min in distilled water. Lead citrate (Reynolds, 1963) and aqueous uranyl acetate (Hayat, 1989) were used to enhance image contrast. Sections were examined using a scanning transmission electron microscope (model 1200EX, JEOL). Immunogold localization experiments were repeated three times, with similar results, using leaves and siliques that had been independently fixed and embedded.

Immunocytological detection of the BCC subunit by light microscopy was carried out by two methods. For immunogold detection, 1-μm-thick London Resin White-embedded tissue sections were placed on glass slides coated with Silane according to the instructions of the manufacturer (Sigma) and dried at just under 50°C for 1 to 12 h. A histo-prep immunomarker (Cel-Tek, Glenview, IL) was used to keep solutions in place over tissue sections. To reduce nonspecific background, sections were incubated overnight in a moist chamber in a suspension of 5% fat-free milk powder in TBS buffer. The suspension was drained off the slides and the sections were briefly blotted.

BCC antiserum (Choi et al., 1995) diluted 1:1000 with TBS-Tween buffer (TBS buffer containing 0.05% [v/v] Tween 20, 0.5% [w/v] BSA, and 0.5% [v/v] normal goat serum) or TBS-Tween only was placed on the resin sections and incubated in a moist chamber at 37°C for 2 h. After incubation, slides were washed with a stream of TBS-Tween buffer and immersed three times for 5 min each

time in TBS-Tween buffer. Ten-nanometer-immunogold-conjugated anti-rabbit IgG diluted 1:100 with TBS-Tween containing 0.1% (w/v) fish gelatin was used as the secondary antibody. Sections were incubated for 2 h at room temperature, washed with a stream of distilled water, immersed five times for 10 min each time in distilled water, and then air-dried. Silver enhancement was carried out using a kit according to the instructions of the manufacturer (Goldmark Biologicals, Phillipsburg, NJ). Twenty-five microliters of the combined initiator and silver-containing solution was placed on sections for 8 to 10 min in the dark. Slides were then washed with a stream of distilled water and immersed twice for 5 min each time in distilled water. Slides were dried at 50°C, immersed in xylene, and coverslipped with Permount (Fisher Scientific). Photographs were taken using an Orthoplan microscope (Leitz) using bright-field, dark-field, and phase-contrast optics and Techpan film (Kodak).

Immunocytological detection of the BCC subunit by PAP was carried out using 7-μm paraffin-embedded sections (John et al., 1992). Sections were pretreated for 30 min with a solution of 1% (w/v) trypsin and immunostained using diaminobenzide as substrate according to the method described in Sternberger (1986). Photographs were taken with an Orthoplan microscope (Leitz) using bright-field optics and Ectachrome T160 film (Kodak).

RESULTS

Isolation and Characterization of the Arabidopsis *CAC1* Gene

The isolation and sequencing of the *CAC1* gene is an initial step that is critical in characterizing the factors that regulate its expression. Southern blot analyses of Arabidopsis genomic DNA indicated that the *CAC1* gene is probably present only once in the genome of Arabidopsis (Choi et al., 1995). The 730-bp fragment from the 3′ end of the *CAC1* cDNA clone was used as a probe to isolate the *CAC1* gene. Approximately 200,000 recombinant bacteriophages from an Arabidopsis genomic library, representing roughly 15 genome equivalents, were screened, resulting in the isolation of three hybridizing clones. Restriction mapping and Southern hybridization analyses of these three clones indicate that they are overlapping and constitute an approximately 25-kb contiguous segment of DNA containing the *CAC1* gene. This finding confirms that the Arabidopsis genome contains a single copy of the *CAC1* gene. The *CAC1* gene is on a 7-kb *Sal*I-*Sac*I fragment that is common to all three of the isolated clones. This *CAC1*-containing fragment was cloned into the plasmid pBSKII(+) for further analysis (we term this clone p6b). Additional restriction mapping, Southern hybridization, and sequencing analyses revealed that the entire coding sequence of the *CAC1* gene is within an approximately 4-kb segment of the Arabidopsis genome.

Comparison of the nucleotide sequence of this 4-kb genomic segment with the *CAC1* cDNA sequence (Choi et al., 1995) led to the identification of the structure of the *CAC1* gene (Fig. 1). The *CAC1* gene is interrupted by six introns with lengths ranging from 84 (intron 2) and 401 bp (intron 1). The seven exons are between 43 (exon 2) and 319 bp (exon 4). The nucleotide sequence of these exons was identical to that of the *CAC1* cDNA (Choi et al., 1995). The amino acid sequence motif that targets the BCC subunit for biotinylation is encoded by exon 5.

The sequences at the intron-exon junctions are similar to the preferred biases observed for other plant genes (Brown, 1989; Ghislain et al., 1994). Namely, plant exon ↓ intron ↓ exon junctions are purine-rich, with a preferred sequence of 5′-AG ↓ GTA-intron-CAG ↓ GT. In the *CAC1* gene the preferred sequences are 5′-yu ↓ G(G/T)G-intron-yAG ↓ u(C/G), in which "y" represents a pyrimidine and "u" represents a purine. The exact 5′ end of exon 1 has not been directly determined, but it must be at or slightly upstream of position −55, which is the 5′ end of the longest *CAC1* cDNA we have sequenced. In addition, a putative TATA box is located on the genomic sequence 132 bp upstream of the ATG translation-initiating codon.

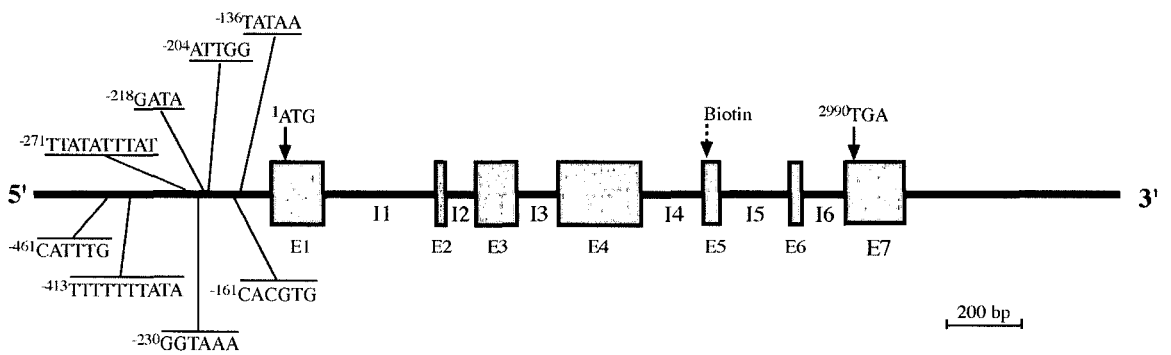

Figure 1. Schematic representation of the structure of the *CAC1* gene of *A. thaliana*. The nucleotide sequence of an approximately 4-kb segment of genomic DNA containing the *CAC1* gene was determined. Exons (E1–E7) are represented by shaded boxes; introns (I1–I6) are represented by solid lines. Positions of the translational start codon (^{1}ATG), stop codon (^{2990}TGA), and biotin attachment site (Biotin) are indicated. Positions of motifs upstream of the first exon that may be important in the regulation of the transcription of the *CAC1* gene are indicated (Gasser and Laemmli, 1986; Guiltinan et al., 1990; Kawagoe and Murai, 1992; Terzaghi and Cashmore, 1995; Allen et al., 1996). Nucleotides are numbered relative to the translational start codon and are shown as superscripts.

Since TATA boxes usually occur between 20 and 40 nucleotides upstream of the transcription start site, the 5′ end of exon 1 may be between positions −110 and −90.

Upstream of the TATA box, beginning at position −698 and extending more upstream of the *CAC1* gene, is the 3′ end of an open reading frame that shows 88% sequence identity with the *Pisum sativum f*-type thioredoxin (GenBank accession number X63537). If this open reading frame is a gene coding for *f*-type thioredoxin, then the promoter of the *CAC1* gene may be contained in less than 600 bases, extending from the 3′ end of the putative thioredoxin *f*-type precursor gene to the start of exon 1. Alternatively, regulatory motifs may overlap the transcribed sequence for the thioredoxin *f*-type gene, which occurs frequently in yeast but to our knowledge has not been reported for plants. The *CAC1* promoter contains several motifs, including the GT-1 binding site, the I-box, and the G-box, shown to be important in light-regulated transcription (Terzaghi and Cashmore, 1995), and the G-box and E-box motifs, shown to be important in specifying high rates of seed-specific transcription (Guiltinan et al., 1990; Kawagoe and Murai, 1992).

The BCC Subunit Is Located in the Stroma of Plastids

The amino acid sequence of the BCC subunit, deduced from the *CAC1* cDNA, contains an NH_2-terminal extension relative to the sequences of bacterial BCC subunits (Choi et al., 1995). This NH_2-terminal extension has characteristics of a transit peptide that would target the BCC subunit to the stroma of plastids. We undertook in situ immunolocalization experiments to provide an independent and direct confirmation of the plastidic localization of the BCC subunit encoded by the *CAC1* gene. Sections from expanding Arabidopsis leaves were reacted first with BCC antiserum and then with anti-rabbit IgG that was conjugated to 10-nm gold particles. Examination of these sections by transmission electron microscopy revealed that the gold particles were located predominantly over the chloroplasts; only a few background gold particles were observed over the cytosol, cell wall, mitochondria, and other cellular compartments (Fig. 2A). In addition, the gold particles were preferentially located over the stromal regions (darker areas) and few were located over the grana (near-white areas). For example, in the figure shown (Fig. 2A), of the 22 gold particles located in the chloroplast, 16 are over the stroma, 5 are either over a region in which the plane of the section of the chloroplast resulted in sectioning through both stroma and grana or over the boundary of the grana and stroma, and 2 are over what appears to be grana. We have seen this pattern consistently with other chloroplasts. Control grids that had been reacted with preimmune serum showed no or very few gold particles over any region of the cells (not shown). These data confirm that the BCC subunit accumulates in leaf chloroplasts, specifically in the stroma.

Cellular Distribution of the BCC Subunit and *CAC1* mRNA in Siliques and Leaves of Arabidopsis

The cellular distribution of the BCC subunit in young, developing leaves was examined by immunocytochemistry using BCC antiserum followed by immunogold localiza-

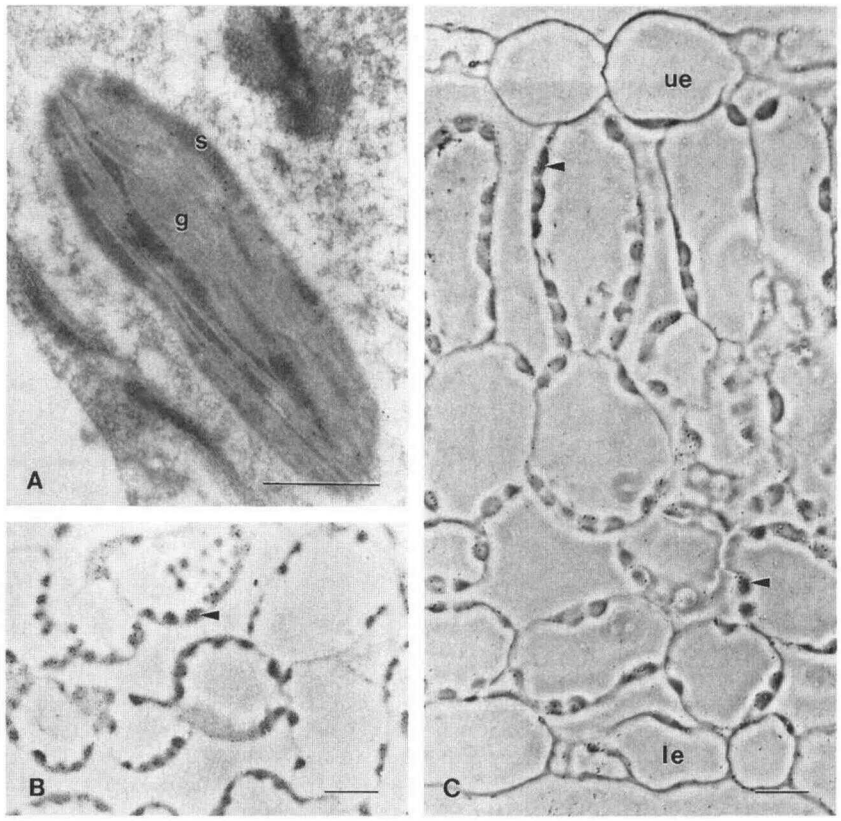

Figure 2. Localization of the BCC subunit in leaves of *A. thaliana*. Expanding leaves were harvested from plants 2 weeks after germination. A, Subcellular localization of BCC subunit in chloroplast stroma. BCC subunit antiserum was used as primary antibody, and anti-rabbit IgG conjugated to 10-nm gold particles was used as a secondary antibody. Electron micrograph of chloroplast and surrounding cytoplasm; grana are lighter regions, and stroma are darker regions within the chloroplast. B, Paradermal section of leaf showing mesophyll cells. C, Cross-section of leaf showing (top to bottom) upper epidermis, palisades parenchyma, spongy mesophyll cells, and lower epidermis. B and C are light micrographs showing immunogold particles visualized by silver enhancement. g, Grana; s, stroma; ue, upper epidermis; and le, lower epidermis. Arrowheads indicate examples of plastids labeled with gold/silver particles. Bar = 0.5 μm in A, 10 μm in B and C.

tion, silver enhancement, and light microscopy. The leaves used in these studies were still expanding and contained cells that were not yet fully differentiated. Both paradermal sections (Fig. 2B) and cross-sections (Fig. 2C) of these leaves were examined. The gold/silver particles representing the location of the BCC subunit were concentrated over the plastids of mesophyll and epidermal cells of the leaf. Control sections using preimmune serum showed no or few silver particles (not shown).

The cellular distribution of the BCC subunit was also examined in young, developing siliques. For these studies we used siliques that were almost fully expanded (at 7 DAF). The embryos within 7-DAF siliques are at the torpedo stage of development and are rapidly depositing seed oils (Mansfield and Briarty, 1992). Two methods were used to immunolocalize the BCC subunit: the higher-resolution immunogold/silver enhancement method (Fig. 3), and a PAP method that produces a brown-colored product (Fig. 4, A and B). As can be seen by the density and distribution of the silver particles (Fig. 3, A and C) and the brown PAP reaction product (Fig. 4A), the BCC subunit accumulates to the highest levels throughout the ground meristem (gm), procambium (pc), and protoderm (p) of the embryo (e) and the cellular endosperm (ce) of the silique. Lower levels of BCC subunit accumulation are detectable in the integumentary tissues (i) (Figs. 3, A and B, and 4A), the cells of the silique central septum (sm) (Figs. 3D and 4A), and the silique wall (w) (Figs. 3, B and E, and 4A). Control sections of embryos treated with preimmune serum and labeled with immunogold/silver enhancement (Fig. 3F) or PAP (Fig. 4B) showed no or little labeling over the plastids of the embryo or other portions of the silique. The brown- and black-staining ring surrounding the cellular endosperm seen in both the immunoreaction (Fig. 4A) and the preimmune control (Fig. 4B) is a material secreted by a subset of integumentary cells, apparently into the vacuoles of these cells. This material, which will become part of the testa, is of undefined composition but contains shikimate derivatives, as indicated by the *clear-testa* mutants (Shirley et al., 1995).

The distribution of *CAC1* mRNA was examined in young, expanding leaves by in situ hybridization to tissue

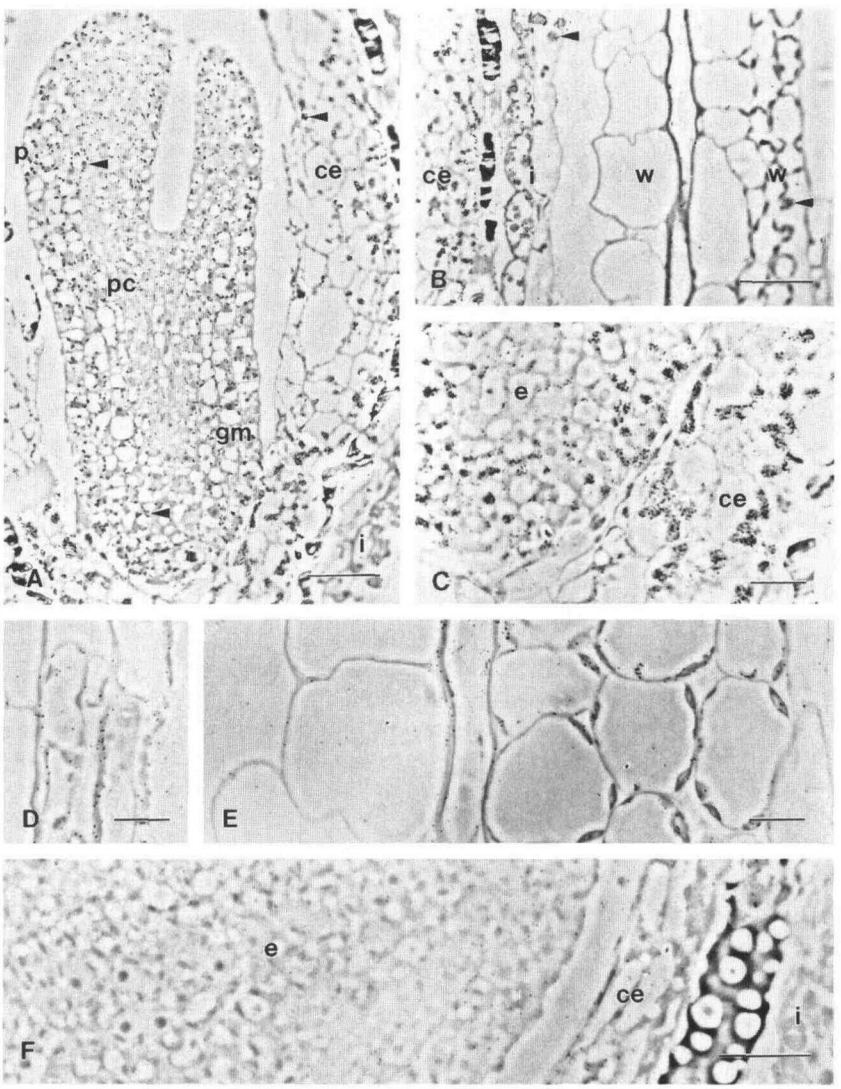

Figure 3. Cellular distribution of BCC subunit in siliques of *A. thaliana*. Developing siliques were harvested 7 DAF. BCC subunit antiserum was used as the primary antibody, and anti-rabbit IgG conjugated to 10-nm gold particles was used as a secondary antibody. Light micrographs showing immunogold particles visualized by silver enhancement. A, Embryo, cellular endosperm, and ovule integument; B, cellular endosperm, integument, and silique wall; C, embryo and cellular endosperm (higher magnification of A); D, central septum; E, silique ovary wall; and F, preimmune control showing embryo, cellular endosperm, and integument. w, Silique wall; i, ovule integument; pc, procambium of embryo; P, protoderm of embryo; gm, ground meristem of embryo; e, embryo; and ce, cellular endosperm. Arrowheads indicate examples of plastids labeled with gold/silver particles. Bar = 30 μm in A and B, and 10 μm in C through F.

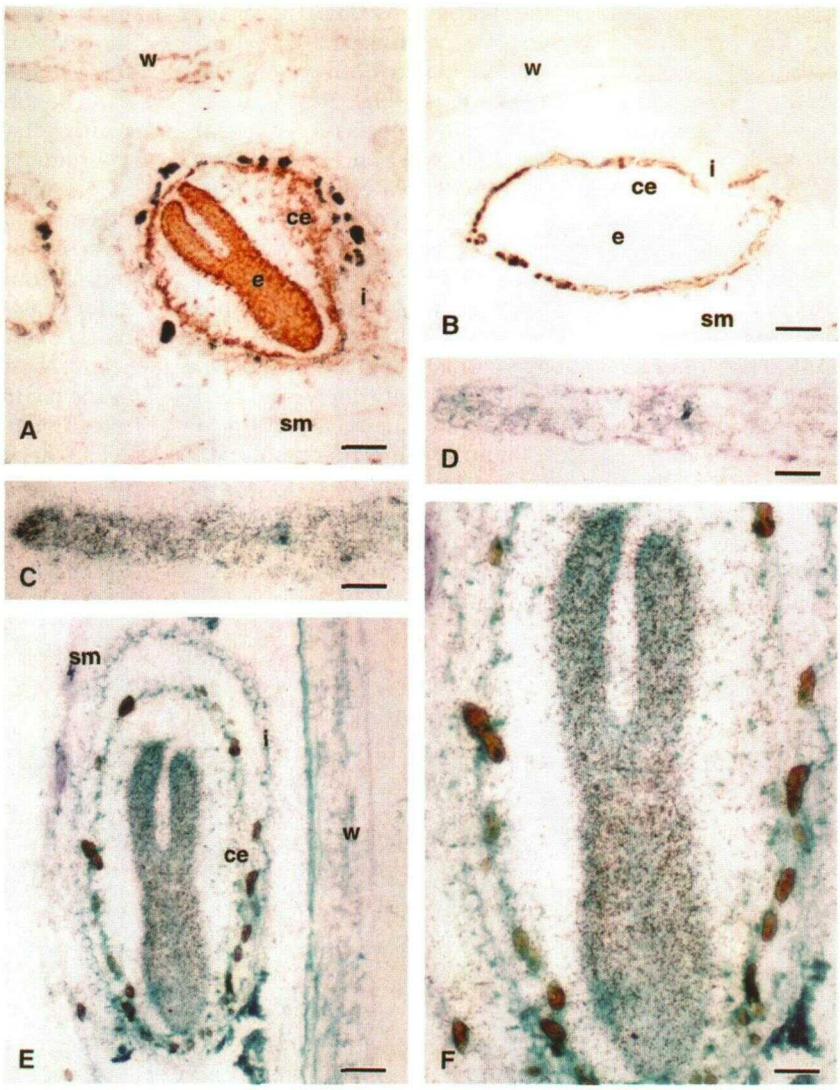

Figure 4. Cellular distribution of BCC subunit and *CAC1* mRNA. Developing siliques of *A. thaliana* were harvested 7 DAF; expanding leaves were harvested from plants 2 weeks after germination. Light micrographs. A, Silique section reacted with BCC subunit antiserum as the primary antiserum and detected by PAP. B, Silique control, similar to A but using preimmune serum as the primary antiserum. Opaque brown-colored material secreted within cells of silique inner integument and surrounding cellular endosperm is not yet fully chemically defined. C, Young leaf. In situ hybridization using antisense RNA probe corresponding to the 3′ end of *CAC1* cDNA. D, Young leaf. In situ hybridization using sense RNA probe corresponding to 3′ end of *CAC1* cDNA. E, Silique. In situ hybridization using antisense RNA probe corresponding to 3′ end of *CAC1* cDNA. F, Silique ovule (higher magnification of E). w, Silique wall; i, ovule integument; e, embryo; ce, cellular endosperm; and sm, central septum. Bar = 59 μm in A through E, and 29 μm in F.

sections using an antisense *CAC1* RNA probe (Fig. 4C). The location of *CAC1* mRNA is visualized by the black-silver grains. The *CAC1* mRNA is present throughout the leaf. Accumulation of *CAC1* mRNA was consistently more pronounced in the cells located near the leaf margins, where growth may have been occurring. Control hybridizations using *CAC1* sense RNA probe show little labeling (Fig. 4D).

The distribution of the *CAC1* mRNA was also determined in developing siliques (7 DAF) (Fig. 4, E and F). The accumulation of *CAC1* mRNA is highest in the embryo, where it is distributed throughout the cells of the cotyledons, root, and shoot axis. *CAC1* mRNA is present at lower levels in the cellular endosperm (ce) and integuments (i) of the developing seed (Fig. 4, E and F). *CAC1* mRNA accumulates only to very low levels in the silique wall (w) and inner septum (sm). Control hybridizations using *CAC1* sense RNA show little labeling over any region of the silique (data not shown).

DISCUSSION

In animals (Vagelos, 1971; Lane et al., 1974; Wakil et al., 1983), yeast (Hasslacher et al., 1993), and bacteria (Kondo et al., 1991; Li and Cronan, 1993), ACCase is a highly regulated enzyme that plays a crucial role in controlling de novo fatty acid biosynthesis. In these organisms a variety of complex mechanisms control ACCase activity. These mechanisms include transcriptional and posttranscriptional regulation of ACCase gene expression, which are generally important for long-term and developmentally controlled changes in rates of fatty acid synthesis. Shorter-term changes in the rate of fatty acid synthesis are generally modulated by alterations of ACCase enzyme activity via kinetic effectors and by covalent modifications.

In plants the role of ACCase in regulating fatty acid biosynthesis is less fully understood. Indirect evidence indicating that ACCase regulates the light-dependence of fatty acid biosynthesis in chloroplasts has been obtained from measurements of acetyl-CoA and malonyl-CoA pools in isolated chloroplasts (Ohlrogge et al., 1993). These modulations of the rates of fatty acid biosynthesis are relatively rapid and are probably the result of kinetic effectors of ACCase activity (Eastwell and Stumpf, 1983; Nikolau and Hawke, 1984). In contrast, less is known about the role of

ACCase in regulating long-term, developmental modulations of fatty acid biosynthesis.

Studies to elucidate this role have compared changes in ACCase activity with changes in fatty acid accumulation during leaf (Nikolau et al., 1981; Hawke and Leech, 1987) and seed (Simcox et al., 1979; Turnham and Northcote, 1982, 1983; Charles et al., 1986; Kang et al., 1994) development. However, these correlative studies are difficult to interpret because, as has now become clear, in vitro measurements of ACCase activity in plant tissue extracts do not necessarily reflect the ACCase that generates malonyl-CoA for fatty acid synthesis. This is for two reasons: (a) Assays of ACCase activity in plant tissue extracts measure the sum of both the cytosolic and plastidic forms of the enzyme, but only the latter generates malonyl-CoA for fatty acid synthesis. Furthermore, the ratio of these two forms of ACCase is not necessarily constant during development. (b) In many plant species (particularly dicots) the plastidic ACCase is heteromeric and is inactivated to an undefined degree by dissociation during extraction (e.g. Alban et al., 1995). Thus, determinations of the activity of heteromeric ACCase may not be quantitative.

To elucidate the role of plastidic ACCase in regulating the long-term developmental changes of fatty acid biosynthesis will require the study of the expression of at least four structural genes, as well as determination of plastidic ACCase activity (which, as described previously, are not yet quantitative). In this study we have focused on the *CAC1* gene. In Arabidopsis, the *CAC1* gene codes for a BCC subunit. Antisera directed against this protein inhibit about 70% of ACCase activity in leaf cell-free extracts, thus indicating that it is a subunit of a heteromeric ACCase (Choi et al., 1995). We previously described a potential transit peptide that could target the BCC subunit encoded by the *CAC1* gene to the plastids. However, plastid-targeting transit peptides also show structural and functional similarities to transit peptides that target proteins to the mitochondria (Keegstra et al., 1989; Heijne, 1992). In addition, the recent finding that plant mitochondria contain an isozyme of acyl-carrier protein (Shintani and Ohlrogge, 1994) may indicate that this organelle may have the ability to carry out de novo fatty acid biosynthesis, as appears to occur in *Neurospora* (Mickolajczyk and Brody, 1990). Here we have used immunohistochemical methods to show that the BCC subunit encoded by the *CAC1* gene is located in the stroma of the plastids, directly demonstrating that this gene encodes the BCC subunit of the plastidic heteromeric ACCase.

The *CAC1* gene is highly expressed in cells that are actively growing and synthesizing fatty acids for membrane phospholipids or triacylglycerides. Thus, the embryos in Arabidopsis siliques at 7 DAF, which are rapidly accumulating seed oils (Mansfield and Briarty, 1992; Bowman, 1994), contain a high level of BCC subunit and *CAC1* mRNA. In contrast, there is less accumulation of BCC subunit and *CAC1* mRNA in the silique walls and inner septum, which have ceased growth but are still metabolically active. The BCC subunit and *CAC1* mRNA are distributed throughout all tissues of the expanding leaf. Our data from siliques and leaves further indicate that the tissue-specific accumulation of the BCC subunit may be controlled primarily by the steady-state accumulation of its mRNA. Thus, in both the siliques and the leaves, the pattern of spatial distribution of the BCC subunit seems to parallel the spatial pattern of distribution of the *CAC1* mRNA that encodes it. These data are consistent with the hypothesis that the developmental pattern of BCC subunit accumulation is determined by the transcriptional regulation of the *CAC1* gene in these organs.

Plastidic ACCase of Arabidopsis is assembled from products of both nuclear and plastidic genes. The plant cell must have mechanisms to coordinate the synthesis, transport, accumulation, and assembly of these subunits. For example, coordination may entail cross-communication among the structural genes during their expression, or the structural genes may be induced by the same set of transcription factors. Comparison of the nucleotide sequence of the *CAC1* promoter with sequences of promoters of nuclear genes coding for the other subunits of the heteromeric ACCase (biotin carboxylase and the α subunit of carboxyltransferase) may provide clues as to how the transcription of these genes is coordinated. This is particularly the case if transcriptional coordination occurs via the binding of a common set of transcription factors that bind to each of these promoter elements. An understanding of the interactive regulatory mechanisms that control the expression of these genes will expand our knowledge of the role of ACCase in the formation of membrane lipids and seed oils.

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LITERATURE CITED

Alban C, Baldet P, Douce R (1994) Localization and characterization of two structurally different forms of acetyl-CoA carboxylase in young pea leaves, of which one is sensitive to aryloxyphenoxyproprionate herbicides. Biochem J **300:** 557–565

Alban C, Julien J, Job D, Douce R (1995) Isolation and characterization of biotin carboxylase from pea chloroplasts. Plant Physiol **109:** 927–935

Allen GC, Hall GE Jr, Michalowski S, Newman W, Spiker S, Weissinger AK, Thompson WF (1996) High level transgene expression in plant cells: effects of a strong scaffold attachment region from tobacco. Plant Cell **8:** 899–913

Bowman J (1994) *Arabidopsis*: An Atlas of Morphology and Development. Springer-Verlag, New York

Brown JW (1989) A catalogue of splice junction and putative branch point sequences from plant introns. Nucleic Acids Res **14:** 9549–9559

Charles DJ, Hasegawa PM, Cherry JH (1986) Characterization of acetyl-CoA carboxylase in the seed of two soybean genotypes. Phytochemistry **25:** 55–59

Choi J-K, Yu F, Wurtele ES, Nikolau BJ (1995) Molecular cloning and characterization of the cDNA coding for the biotin-containing subunit of the chloroplastic acetyl-CoA carboxylase. Plant Physiol **109:** 619–625

Conn EE (1981) The Biochemistry of Plants: A Comprehensive Treatise, Vol 7. Secondary Plant Metabolism. Academic Press, New York

Eastwell K, Stumpf PK (1983) Regulation of plant acetyl-CoA carboxylase by adenylate nucleotides. Plant Physiol **72:** 50–55

Egin-Buhler B, Loyal R, Ebel J (1980) Comparison of acetyl-CoA carboxylase from parsley cell cultures and wheat germ. Arch Biochem Biophys **203:** 90–100

Egli MA, Gengenbach BG, Gronwald JW, Somers DA, Wyse DL (1993) Characterization of maize acetyl-coenzyme A carboxylase. Plant Physiol **101:** 499–506

Gasser SM, Laemmli UK (1986) Cohabitation of scaffold binding regions with upstream enhancer elements of three developmentally regulated genes of *D. melanogaster*. Cell **46:** 521–530

Ghislain M, Frankard V, Vandenbossche D, Matthews BF, Jacobs M (1994) Molecular analysis of the aspartate kinase-homoserine dehydrogenase gene from *Arabidopsis thaliana*. Plant Mol Biol **24:** 835–851

Gornicki P, Podkowinski J, Scappino LA, DiMaio J, Ward E, Haselkorn R (1994) Wheat acetyl-coenzyme A carboxylase: cDNA and protein structure. Proc Natl Acad Sci USA **91:** 6860–6864

Gornicki P, Scappino LA, Haselkorn R (1993) Genes for two subunits of acetyl coenzyme A carboxylase of *Anabaena* sp. strain PCC 7120: biotin carboxylase and biotin carboxyl carrier protein. J Bacteriol **175:** 5268–5272

Guchhait RB, Polakis SE, Dimroth P, Stoll E, Moss J, Lane MD (1974) Acetyl coenzyme A carboxylase system of *Escherichia coli*. J Biol Chem **249:** 6633–6645

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A leucine zipper protein that recognizes an abscisic acid response element. Science **250:** 267–270

Harwood JL (1988) Fatty acid metabolism. Annu Rev Plant Physiol Plant Mol Biol **39:** 101–138

Hasslacher M, Ivessa AS, Paltauf F, Kohlwein SD (1993) Acetyl-CoA carboxylase from yeast is an essential enzyme and is regulated by factors that control phospholipid metabolism. J Biol Chem **268:** 10946–10952

Hawke JC Leech RM (1987) Acetyl-CoA carboxylase activity in normally developing wheat leaves. Planta **171:** 489–495

Hayat MA (1989) Principles and Techniques of Electron Microscopy: Biological Applications, Ed 3. Macmillan, London

Heijne GV (1992) Cleavage-site motifs in protein targeting sequences. Genet Eng **14:** 1–11

John I, Wang H-Q, Held BM, Wurtele ES, Colbert JT (1992) An mRNA that specifically accumulates in maize roots delineates a novel subset of developing cortical cells. Plant Mol Biol **20:** 821–831

Kang F, Ridout CJ, Morgan CL, Rawsthorne S (1994) The activity of acetyl-CoA carboxylase is not correlated with the rate of lipid synthesis during development of oilseed rape (*Brassica napus* L.) embryos. Planta **193:** 320–325

Kannangara CG, Stumpf PK (1972) Fat metabolism in higher plants. LIV. Prokaryotic type acetyl-CoA carboxylase in spinach chloroplasts. Arch Biochem Biophys **152:** 83–91

Kawagoe Y, Murai N (1992) Four distinct nuclear proteins recognize *in vitro* the proximal promoter of the bean seed storage protein β-phaseolin gene conferring spatial and temporal control. Plant J **2:** 927–936

Keegstra K, Olsen LJ, Theg SM (1989) Chloroplastic precursors and their transport across the envelope membranes. Plant Mol Biol **40:** 471–501

Kondo H, Shiratsuchi K, Yoshimoto T, Masuda T, Kitazono A, Tsuru D, Anai M, Sekiguchi M, Tanabe T (1991) Acetyl-CoA carboxylase from *Escherichia coli*: gene organization and nucleotide sequence of the biotin carboxylase subunit. Proc Natl Acad Sci USA **88:** 9730–9733

Konishi T, Sasaki Y (1994) Compartmentalization of two forms of acetyl-CoA carboxylase in plants and the origin of their tolerance toward herbicides. Proc Natl Acad Sci USA **91:** 3598–3601

Lane MD, Moss J, Polakis SE (1974) Acetyl coenzyme A carboxylase. Curr Top Cell Regul **8:** 139–195

Li S-J, Cronan JE Jr (1992a) The gene encoding the biotin carboxylase subunit of *Escherichia coli* acetyl-CoA carboxylase. J Biol Chem **267:** 855–863

Li S-J, Cronan JE Jr (1992b) The genes encoding the two carboxyltransferase subunits of *Escherichia coli* acetyl-CoA carboxylase. J Biol Chem **267:** 16841–16847

Li S-J, Cronan JE Jr (1993) Growth rate regulation of *Escherichia coli* acetyl coenzyme A carboxylase, which catalyzes the first committed step of lipid biosynthesis. J Bacteriol **175:** 332–340

Lopez-Casillas F, Bai D-H, Luo X, Kong I-S, Hermodson MA, Kim K-H (1988) Structure of the coding sequence and primary amino acid sequence of acetyl-coenzyme A carboxylase. Proc Natl Acad Sci USA **85:** 5784–5788

Mansfield SG, Briarty LG (1992) Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. Can J Bot **70:** 151–164

Mickolajczyk S, Brody S (1990) De novo fatty acid synthesis mediated by acyl carrier protein in *Neurospora crassa* mitochondria. Eur J Biochem **187:** 431–437

Nikolau BJ, Hawke C (1984) Purification and characterization of maize leaf acetyl-coenzyme A carboxylase. Arch Biochem Biophys **228:** 86–96

Nikolau BJ, Hawke JC, Slack CR (1981) Acetyl-coenzyme A carboxylase in maize leaves. Arch Biochem Biophys **211:** 605–612

Nikolau BJ, Wurtele ES, Stumpf PK (1984) Tissue distribution of acetyl-coenzyme A carboxylase in leaves. Plant Physiol **75:** 895–901

Ohlrogge JB, Jaworski JG, Post-Beittenmiller D (1993) De novo fatty acid biosynthesis. *In* TS Moore, ed, Lipid Metabolism in Plants, CRC Press, Boca Raton, FL pp 3–32

Ohlrogge JB, Kuhn DN, Stumpf PK (1979) Subcellular localization of acyl carrier protein in leaf protoplasts of *Spinacia oleracea*. Proc Natl Acad Sci USA **76:** 1194–1198

Pollard M, Stumpf PK (1980) Biosynthesis of C20 and C22 fatty acids by developing seeds of *Limnanthes alba*. Plant Physiol **66:** 649–655

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol **17:** 208–212

Roesler KR, Shorrosh BS, Ohlrogge JB (1994) Structure and expression of an *Arabidopsis* acetyl-coenzyme A carboxylase gene. Plant Physiol **105:** 611–617

Roessler PG, Ohlrogge JB (1993) Cloning and characterization of the gene that encodes acetyl-coenzyme A carboxylase in the alga *Cyclotella cryptica*. J Biol Chem **268:** 19254–19259

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Sasaki Y, Hakamada K, Suama Y, Nagano Y, Furusawa I, Matsuno R (1993) Chloroplast-encoded protein as a subunit of acetyl-CoA carboxylase in pea plant. J Biol Chem **268:** 25118–25123

Sasaki Y, Konishi T, Nagano Y (1995) The compartmentation of acetyl-coenzyme A carboxylase in plants. Plant Physiol **108:** 445–449

Schulte W, Schell J, Topfer R (1994) A gene encoding acetyl-coenzyme A carboxylase from *Brassica napus*. Plant Physiol **106:** 793–794

Shintani DK, Ohlrogge JB (1994) The characterization of a mitochondrial acyl carrier protein isoform isolated from *Arabidopsis thaliana*. Plant Physiol **104:** 1221–1229

Shirley BW, Kubasek WL, Storz G, Bruggemann E, Korneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. Plant J **8:** 659–671

Shorrosh BS, Dixon RA, Ohlrogge JB (1994) Molecular cloning, characterization, and elicitation of acetyl-CoA carboxylase from alfalfa. Proc Natl Acad Sci USA **91:** 4323–4327

Shorrosh BS, Roesler KR, Shintani D, van de Loo FL, Ohlrogge JB (1995) Structural analysis, plastid localization, and expression of the biotin carboxylase subunit of acetyl-coenzyme A carboxylase from tobacco. Plant Physiol **108:** 805–812

Shorrosh BS, Savage LJ, Soll J, Ohlrogge JB (1996) The pea chloroplast membrane-associated protein, IEP96, is a subunit of acetyl-CoA carboxylase. Plant J **10:** 261–268

Simcox PD, Garland W, DeLuca V, Canvin VT, Dennis DT (1979) Respiratory pathway and fat synthesis in the developing castor bean oil seed. Can J Bot **57:** 1008–1014

Sternberger LA (1986) Immunocytochemistry, Ed 2. John Wiley & Sons, New York

Terzaghi WB, Cashmore AR (1995) Light regulated transcription. Annu Rev Plant Physiol Plant Mol Biol **46:** 445–474

Turnham E, Northcote DH (1982) The use of acetyl-CoA carboxylase activity and changes in wall composition as measures of embryogenesis in tissue cultures of oil palm (*Elaeis guineensis*). Biochem J **208:** 323–332

Turnham E, Northcote DH (1983) Changes in the activity of acetyl-CoA carboxylase during rape-seed formation. Biochem J **212:** 223–229

Vagelos RP (1971) Regulation of fatty acid biosynthesis. Curr Top Cell Regul **4:** 119–166

Voytas DF, Konieczny A, Cummings MP, Ausubel FM (1990) The structure, distribution and evolution of the Ta*1* retrotransposable element family of *Arabidopsis thaliana*. Genetics **126:** 713–721

Wakil SJ, Stoop JK, Joshi VC (1983) Fatty acid synthesis and its regulation. Annu Rev Biochem **52:** 537–579

Walid A-F, Chirala SS, Wakil SJ (1992) Cloning of the yeast *FAS3* gene and primary structure of yeast acetyl-CoA carboxylase. Proc Natl Acad Sci USA **89:** 4534–4538

Wang H-Q, Colbert JT, Wurtele ES (1995) A molecular marker of the root cortical ground meristem is present in both root and coleorhiza of the maize embryo. Am J Bot **82:** 1083–1088

Yanai Y, Kawasaki T, Shimada H, Wurtele ES, Nikolau BJ, Ichikawa N (1995) Genomic organization of 251 kDa acetyl-CoA carboxylase genes in *Arabidopsis*: tandem gene organization has made two differentially expressed isozymes. Plant Cell Physiol **36:** 779–787